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Heat shock and UV-B episodes modulate olive leaves lipophilic and phenolic metabolite profiles

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ABSTRACT

Olea europaea L. is the basis of the olive oil chain value. Olive by-products remain economically underexplored despite their richness in polyphenols and fatty acids. Heat waves and raised UV-B radiation episodes (that increasingly affect Mediterranean orchards) may influence these compounds. Herein we characterize the most relevant leaf lipophilic and phenolic compounds of an important Portuguese cultivar Cobrançosa, immediately and 30 days after exposure to high UV-B radiation or heat shock (HS). Plants were exposed to either UV-B (12 kJ m^{-2} d^{-1}) or HS (40 °C for 2 h) for two consecutive days. Hydroxycinnamic acids, triterpenes, sterols and fatty acids are less influenced by UV-B and HS, while the amounts of flavonoids, secoiridoids and monoterpenes increased. In particular, after both treatments, quercetin-3-*O*-glucoside levels increased in leaves, and UV-B stimulated oleuropein levels. HS reduced the amounts of verbascoside, oleuropein and luteolin-7-*O*-glucoside, while UV-B only decrease apigenin-7-*O*-rutinoside levels. Thirty days after UV-B and HS relief, these compounds showed different profiles. Whereas UV-B recovering plants showed increased amounts of thymol-β-D-glucopyranoside, in HS recovering plants, the increase was more relevant for oleuropein, chrysoeriol-7-*O*-glucoside and luteolin-7-*O*-glucoside. Data evidence that Cobrançosa leaves are particularly rich in luteolin-7-*O*-glucoside, oleuropein, and oleic and palmitic acids, and that their levels may be stimulated by climate-change related conditions, contributing to the economical valorisation of leaves.

1. Introduction

During the last three decades the high interest and consumption of olive oil and olives promoted the widespread of olive tree (*Olea europaea* L.) to countries like Australia, China, India and South America (Torres et al., 2017), but its cultivation is mainly (˜98%) in the Mediterranean basin. Spain, Italy and Greece are the main olive oil producers (˜77%), followed by Tunisia, Syria, Turkey, Morocco and Portugal (International Olive Council, 2018).

Despite the high economic and health value of olives and olive oil, the interest of some olive and olive oil industry by-products (e.g. leaves, stones) have been increasing (Williams et al., 2017; del Pozo et al., 2018; Lammi et al., 2018). Leaf by-products may be used to exploit their richness in polyphenols, fatty acids, terpenes and minerals, while addressing the needs of a circular economy (Guinda et al., 2015). Historically, olive leaves have been used in folk medicine (Benavente-García et al., 2000) and leaf-extracts exhibit a large bioactive properties including antioxidant, anti-inflammatory, antimicrobial and antitumor/cancer (Talhaoui et al., 2015). Therefore, the use of these compounds for the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceuticals and cosmetic products is in broad expansion (Romani et al., 2017).

The health benefits of olive leaf compounds are mostly related to antioxidants such as polyphenols that may protect the organism against oxidative damages (e.g. by neutralizing free radicals; Rigacci and Stefani, 2016). In the food industry, these compounds are usually used as food additives to inhibit lipid oxidation preventing product deterioration (Romani et al., 2017). The most abundant polyphenols include secoiridoids, flavonoids and simple phenolic compounds (Leouifoudi et al., 2014; Talhaoui et al., 2015). Among secoiridoids, which are the

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main family of compounds in leaves, oleuropein is the most abundant bioactive compound (Irakli et al., 2018). Flavonoids (e.g., luteolin-7-*O*-glucoside, quercetin-7-*O*-rutinoside and apigenin-7-*O*-glucoside) occur in appreciable amounts in leaves while the simple phenols (e.g. hydroxytyrosol and tyrosol) and phenolic acids (e.g. verbascoside and caffeic acid) are less representative in olive leaves but significantly contribute to the overall leaf antioxidant potency (Taamalli et al., 2012).

Few studies have addressed other bioactive compounds with nutritional value, such as fatty acids and terpenes. Most of the health beneficial properties of olive oil are related to the presence of fatty acids such as palmitic, oleic, linoleic and stearic, and leaves are also rich in these compounds (Cavalheiro et al., 2015; Dias et al., 2018). Additionally, high contents of terpenes (e.g. thymol, ursolic acid, amyrin and lupeol derivatives) with relevant antioxidant activities, as well as amides (oleamide) (Guinda et al., 2015; Dias et al., 2018) and sterols were also identified in olive leaves. In addition to the above-mentioned phenolic compounds these lipophilic compounds also contribute to highlight the important industrial application of olive leaves.

Several factors, such as the olive cultivar/genotype, developmental stage and environmental factors may influence the qualitative and quantitative composition of olive leaves (Rigacci and Stefani, 2016). Among the environmental factors, stressors related to climate change (e.g. heat, high UV-B radiation and drought) changed phenolic and lipophilic profile of leaves, fruits and olive oil and also impacted olive productivity (Palese et al., 2010; Petridis et al., 2012; Martinelli et al., 2013; Cetinkaya et al., 2016; Dias et al., 2018). Taking into consideration that the emerging climate change scenarios occurred in a global scale, predictably more pronounced in the Mediterranean region (IPCC: Field et al., 2014), the future impact and change on leaf/olive/oils metabolite patterns raises an emergent concern and discussion.

This study was undertaken to investigate the impacts of heat and high UV-B radiation shocks on the most representative olive leaf phenolic and lipophilic compounds levels. This study also aims at deciphering how these climate change related stressors may enrich leaves with bioactive compounds, promoting their commercial exploitation.

2. Material and methods

2.1. Plant material, culture conditions, and heat and UV-B treatments application

Potted (2 L black plastic pots with a sandy-loam soil 2:1, v/v) *Olea europaea* L. plants (cv Cobrançosa) seven months old provided by Viveiros Vitor Costa (Mealhada, Portugal) were acclimated for 3 months to a temperature of 23 ± 2 °C, relative humidity (RH) of 40 ± 5% and a daylight photoperiod of 16/8 h in a climate chamber. The light in the climate chamber was provided by Osram cool white fluorescent lamps with an intensity of 600 μmol m^{-2} s^{-1}. One day before the treatments, plants with similar height (73 ± 4 cm) were randomly organised in three groups and for each plant the youngest fully expanded leaves (that correspond to the ~ 5th node from the top) was marked. In group one – control (C, n = 20), plants were maintained under ideal growth conditions (climate chamber conditions described above); in group two – heat shock (HS, n = 20), plants were exposed to increased temperature for two consecutive days; and in group three – UV-B shock (UV-B, n = 16), plants were exposed to an effective UV-B dose of 12 kJ m^{-2} d^{-1}, for two consecutive days. The heat shock (HS treatment) was carried out in a climate chamber (ARALAB - Fitoclima 4600) with the same RH, photoperiod and light intensity conditions. The HS treatment was performed as followed: temperature was gradually elevated from 23 °C to 31 °C during 1 h, followed by the increase from 31 °C to 40 °C also during 1 h, and then plants were kept at 40 °C for 2 h. The UV-B treatment was performed with a light system composed of ten UV-B lamps with an emission spectrum of 280–320 nm and maximum emission at 306–312 nm (Sankyo Denki G8T5E, Kanagawa, Japan), equipped with a borosilicate glass filter to block radiation below 290 nm (UV-C radiation ~0 kJ m^{-2}) as described in Dias et al. (2018). UV-B biologically effective dose used, 12 kJ m^{-2} d^{-1}, was chosen according to Dias et al. (2018) data and calculated according to Correia et al. (2012). UV-B irradiance homogeneity was assessed daily using a VLX calibrated radiometer (VLX 254 and VLX 312, Vilber Lourmat, Marne-la-Vallée Cedex, France), and the spectral sensitivity and corresponding correction factor determined (Correia et al., 2012). Immediately after treatments, leaves from the marked nodes (from the 5th to the 7th nodes) were collected in half of the plants of each group (C group n = 10; HS group n = 10; and UV-B group n = 8), frozen in liquid nitrogen and stored at -80 °C. The other half of the plants (C group n = 10; HS group n = 10; and UV-B group n = 8) were kept under the optimal growth condition and used to evaluate the plant recovery capacity 30 days after stress relief. Leaves sampling was taken as described above.

2.2. Preparation of extracts and chromatography analysis

2.2.1. Extract preparation

For each treatment (C, HS or UV-B) olives leaves were grinded in a small mill (only used for olives) to get a powder that was first used to metabolite extraction with *n*-hexane and then with ethanol. Leaf powder from plants of each treatment were separately extracted with *n*-hexane, at room temperature with magnetic stirring for 72 h. After the first extraction, the *n*-hexane was renewed two more times and the extraction continued for 72 h. The *n*-hexane resulting from the extractions was evaporated to dryness at low pressure and the dried extracts were analysed by gas chromatography-mass spectrometry (GC–MS). The resulting pellet from *n*-hexane was used to extract phenolic compounds. The pellet from each treatment was extracted with hot ethanol at constant heating (Soxhlet method). After the first 72 h, ethanol was renewed two more times and the extraction continued for more 72 h. After 9 days, the ethanol resulting from the extractions was evaporated to dryness at low pressure and the dried extracts were analysed by ultra-high performance liquid chromatography – mass spectrometry (UHPLC-MS).

2.2.2. GC–MS analyses

Before injections, the extracts from all treatments were silylated. In a glass tube, 250 μL of pyridine, 250 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide, 50 μL of trimethylsilyl chloride, 200 μL of the leaf extract (~100 mg mL^{-1}) and 200 μL of the internal standard (tetracosane, 0.465 mg mL^{-1}) were mixed and placed in a water bath at 70 °C for 30 min. GC–MS analyses of each extract silylated (from the three treatments, C, HS and UV-V) were performed using a GC–MS QP2010 Ultra Shimadzu equipped with a DB-5- J&W capillary column (30 m × 0.25 mm id and a film thickness of 0.25 μm). Helium was used as carrier gas (35 cm s^{-1}). Initial temperature was set to 80 °C for 5 min and temperature rate, 4 °C min^{-1} up to 285 °C, was maintained for 10 min. The injector temperature was 250 °C and the transfer-line temperature was 290 °C, with a split ratio of 1:50. The mass spectrometer was operated in the electron impact (EI) mode with energy of 70 eV and data were collected at a rate of 1 scan s^{-1} (m/z 33–750). The ion source was maintained at 250 °C. The obtained chromatographic peaks were identified by comparison with library entries of mass spectra database (NIST14 Mass spectral and WILEY RegistryTM of Mass Spectra Data) and/or with retention times and mass spectra data of the standard compounds (prepared and injected in the same chromatographic conditions). For quantification analysis, GC–MS was calibrated with pure standard compounds (palmitic acid and cholesterol) representative of the major lipophilic compounds relative to the internal standard (tetracosane) after silylation.

2.2.3. UHPLC-MS analyses

Approximately 1 g of each extract was dissolved in 10 mL of

methanol (10 mg mL^{-1}). Samples were filtered (0.2 mL nylon membrane, Whatman) and injected in a UHPLC-MS (Thermo Scientific Ultimate 3000RSLC Dionex). The equipment contain a Dionex UltiMate 3000 RS diode array detector coupled to a mass spectrometer. A thermoscientific hypersil gold column (1000 mm × 2.1 mm) with a part size of 1.9 µm was used and the temperature set to 30 °C. The mobile phase consists of degassed and filtered acetonitrile (A) and 0.1% formic acid (B) (v/v) with a flow rate of 0.2 mL min^{-1}. Solvent gradient was initiated with 5% of solvent B over 14 min proceeded by 40% of solvent B for 2 min, 100% over 7 min, and 5% over the last 10 min. 2 µL were injected to the equipment. UV–vis spectral data were collected (250–500 nm) and the chromatographic profiles documented at 280 nm. A mass spectrometer (LTQ XL linear ion trap 2D) equipped with an orthogonal electrospray ion source (ESI), operated in a negative-ion mode with electrospray ionization source of 5.00 kV (ESI capillarity temperature of 275 °C) was used. A mass range of 50.00–2000.00 *m/z* was covered and collision-induced dissociation MS/MS and MS^n experiments were simultaneously acquired for precursor ions.

The phenolic compounds identification was carried out by comparison of the retention times, UV–vis spectra and spectra data with those of the closest available reference standards and data reported in the literature. Semi-quantification of phenolic compounds was achieved by peak integration (280 nm), through the external standard method, using the closest reference compound available. The detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves. The calibration curves were performed by injection of six known concentrations (with variable ranges: 1–1000 µg mL^{-1}) of reference compounds (quercetin and caffeic acid). The concentrations of these reference compounds were selected to guarantee the quantification of each compound in the samples by intrapolation in the calibration curve. Correlation coefficient values confirmed linearity of the calibration plots ($r^2 > 0.98$). The results were expressed in g of compound per kg^{-1} of leaf DW of three independent analyses per treatments.

2.3. Statistical analysis

Data were analysed by one-way analysis of variance. When significant differences were found the Holm-Sidak Comparison Test was performed. The significance level was 0.05. Statistical analysis was performed in the SigmaStat program for Windows, version 3.1 (Systat Software, San Jose, CA) and heat map analysis was performed in excel program (Windows 10).

3. Results

3.1. Phenolic profile

The phenolic profile was assessed in olive leaves from plants under Control, HS and UV-B conditions and is presented in Table 1. Overall, nine compounds were quantified: five flavonoids (one monohydroxy B-ring-substituted flavonoid the quercetin-3-*O*-glucoside and four *o*-dihydroxy B-ring-substituted flavonoids), three secoiridoids and one hydroxycinnamic acid derivative. Quantitative changes were observed among conditions. Flavonoids and secoiridoids were the main representative chemical classes identified in all treatments.

Immediately after stress exposure the sum of flavonoids quantities varied from 2.807 g kg^{-1} DW in C plants to 2.294 g kg^{-1} DW in HS-treated plants. The analysis of the flavonoids separately showed that both treatments improved significantly (˜2 × more than control) quercetin-3-*O*-glucoside levels. On the other hand, the apigenin-7-*O*-rutinoside in the UV-B treatment and the luteolin-7-*O*-glucoside in the HS treatment decreased significantly. Other flavonoids, chrysoeriol-7-*O*-glucoside and quercetin-3-*O*-rutinoside were not significantly affected by the HS and UV-B treatments. Thirty days after stress relief a

Table 1
Phenolic composition (g kg^{-1} DW) of olive leaves from control (C), heat-shock (HS) and UV-B (UV-B) treatments immediately after experiment and 30 days after stress (HS and UV-B) relief. Compounds characterized using the negative ionisation mode. Values are given as mean ± standard deviation (n = 3–4). For each line and condition (stress exposure or stress relief), different letters indicate significant differences ($p < 0.05$). Retention time (Rt) is represented in minutes.

Rt	Compound	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*) fragments	Stress Exposure			Stress Relief		
				C	HS	UV-B	C	HS	UV-B
Flavonoids									
12.0	Quercetin-3-*O*-rutinoside	609	301	0.123 ± 0.014 ^{a}	0.104 ± 0.010 ^{a}	0.109 ± 0.010 ^{a}	0.517 ± 0.171 ^{a}	0.779 ± 0.090 ^{a}	0.653 ± 0.136 ^{a}
12.4	Quercetin-3-*O*-glucoside	463	301; 300	0.035 ± 0.010 ^{b}	0.072 ± 0.012 ^{a}	0.074 ± 0.002 ^{a}	0.066 ± 0.005 ^{a}	0.066 ± 0.007^{a}	0.064 ± 0.027 ^{a}
12.5	Apigenin-7-*O*-rutinoside	577	269	0.246 ± 0.051 ^{a}	0.194 ± 0.018 ^{a}	0.116 ± 0.035 ^{b}	0.126 ± 0.042 ^{a}	0.041 ± 0.013 ^{b}	0.051 ± 0.021 ^{b}
12.6	Luteolin -7-*O*-glucoside	447	285	2.164 ± 0.094 ^{a}	1.702 ± 0.010 ^{b}	2.193 ± 0.063 ^{a}	2.054 ± 0.386 ^{b}	3.449 ± 0.361 ^{a}	2.118 ± 0.120 ^{b}
14.1	Chrysoeriol-7-*O*-glucoside	461	299; 446	0.239 ± 0.036 ^{a}	0.222 ± 0.017 ^{a}	0.212 ± 0.013 ^{a}	0.333 ± 0.064 ^{b}	0.645 ± 0.164 ^{a}	0.499 ± 0.014 ^{ab}
Secoiridoids									
14.3	Methyloleuropein	553	391; 359	0.199 ± 0.060 ^{a}	0.241 ± 0.007 ^{a}	0.190 ± 0.010 ^{a}	0.182 ± 0.010 ^{b}	0.231 ± 0.012 ^{a}	0.180 ± 0.010 ^{b}
14.4	2"-Methoxyoleuropein	568	537; 403; 407	0.175 ± 0.012 ^{b}	0.161 ± 0.022 ^{b}	0.229 ± 0.010 ^{a}	0.165 ± 0.013 ^{b}	0.162 ± 0.012 ^{b}	0.220 ± 0.011 ^{a}
14.5	Oleuropein	539	377; 307; 275	1.255 ± 0.142 ^{b}	0.933 ± 0.077 ^{c}	1.648 ± 0.081 ^{a}	1.363 ± 0.188 ^{b}	2.347 ± 0.180 ^{a}	1.300 ± 0.047 ^{ab}
Hydroxycinnamic Acid Derivatives									
12.7	Verbascoside	623	461	0.239 ± 0.029 ^{a}	0.120 ± 0.005 ^{b}	0.288 ± 0.027 ^{a}	0.103 ± 0.022 ^{a}	0.064 ± 0.010 ^{a}	0.074 ± 0.019 ^{a}

general inversion of response was observed: the sum of flavonoids quantities in plants exposed to stress was higher than in C plants. Compared to C plants, the flavonoids luteolin-7-*O*-glucoside and chryoeriol-7-*O*-glucoside increased ($p < 0.05$) after HS treatment. Apigenin-7-*O*-rutinoside remained lower ($p < 0.05$) in the UV-B treatment, and even a reduction ($p < 0.05$) in the HS treatment was observed when compared to plants under C conditions.

As far as the sum of secoiridoids quantities in olive leaves is concerned, HS promoted immediately its decrease (18%) mostly due to the reduction ($p < 0.05$) of oleuropein, while UV-B treatment increased their levels (18%) due to the augment ($p < 0.05$) of oleuropein and 2″-methoxyoleuropein levels. Thirty days after stress relief, an opposite profile of secoiridoids was observed in plants previously exposed to HS with the sum of quantities of these compounds increasing mostly due to oleuropein and methyloleuropein. The levels of secoiridoids in plants previously exposed to UV-B were maintained, despite the significant increase of 2″-methoxyoleuropein.

For the hydroxycinnamic acid derivatives group, only verbascoside was identified and quantified. This compound only significantly decreased immediately after HS exposure (50% reduction compared to control). Thirty days after stress relief, a tendency for verbascoside reduction (38% in HS and 28% in UV-B plants) was observed, despite not significant.

3.2. Lipophilic profile

The lipophilic profile was assessed in olive leaves from plants under Control, HS and UV-B conditions and is presented in Table 2. Overall, ten compounds were identified: two fatty acids, six terpenes and two sterols. Quantitative changes were observed among treatments. Fatty acids are one of the main chemical class of compounds in olive leaves from all treatments. One saturated (palmitic acid) and one unsaturated fatty acid (oleic acid) were identified in leaves of the cultivar Cobrançosa. Immediately after stress application, the sum of fatty acids quantities varied from 1.343 g kg^{-1} DW in control and UV-B treated plants, to 0.960 g kg^{-1} DW in HS plants, thus showing a significant decrease with HS treatment. Thirty days after stress removal, besides plants from HS treatment also those from the UV-B treatment presented a sum of fatty acids quantities lower (1.306 and 1.372 g kg^{-1} DW, respectively) than in control plants (2.671 g kg^{-1} DW). The ratio of saturated to unsaturated fatty acids (palmitic acid/oleic acid) varied from 0.97 to 1.00 during the stress exposure period in all treatments, to 0.80-0.86 during the stress relief period in all treatments.

Terpenes represent another major class of chemical compounds. Within this class, one monoterpene (thymol-β-D-glucopyranoside) and five triterpenes (α- and β-amyrin, lupeol derivatives and ursolic acid) were identified in olive leaves. The sum of terpenes quantities in olive leaves varied from 0.607 g kg^{-1} DW in HS treated plants to 0.956 g kg^{-1} DW in UV-B treated plants. In general, HS treatment induced a stronger decrease in the levels of these compounds, mostly due to the reduction ($p < 0.05$) of α-amyrin and ursolic acid. After stress relief, plants from the HS treatment continued, in general, to show a lower level of terpenes (except for the case of thymol-β-D-glucopyranoside that was not affected). For the case of the plants previously exposed to UV-B treatment, only the content of thymol-β-D-glucopyranoside increased significantly, but in general the levels of the other terpenes identified were below the control ones.

Two sterols were identified and quantified in olive leaves, the stigmasterol and β-sitosterol. Their levels ranged from 0.213-0.307 g kg^{-1} DW immediately after stress application to 0.244-0.420 g kg^{-1} DW after stress relief. Immediately after stress application, stigmasterol content decreased significantly only in HS treated plants. Thirty days after stress relief, sterols decrease ($p < 0.05$) was more accentuated in plants previously exposed to HS treatment, and lower levels of sterols were observed in plants previously exposed to UV-B treatment.

4. Discussion

Despite the great economic importance of olives and olive oil, olive leaves represent an emergent important source of several bioactive compounds with interesting health properties and wide industrial applications (Romani et al., 2017; del Pozo et al., 2018). In Cobrançosa leaves the flavonoids and secoiridoids are the main phenolic compounds (followed by fatty acids), being within the ranges (~0.5–10 g kg^{-1} DW) described for other cultivars (Talhaoui et al., 2015). Unlikely other cultivars (Quirantes-Piné et al., 2013; Romani et al., 2017), luteolin-7-*O*-glucoside, followed by oleuropein, are the most representative identified compounds in Cobrançosa. Other secoiridoids (e.g. ligstroside and oleuroside) and the flavonoid diosmetin were not identified in Cobrançosa, although abundant in leaves of Arbequina, Hojiblanca, Picual and Sikitita (Quirantes-Piné et al., 2013; Talhaoui et al., 2015; Jiménez-Sánchez et al., 2017).

UV-B-induces the biosynthesis of some flavonoids, increasing the ratio of the "effective antioxidants" (*o*-dihydroxy B-ring-substituted flavonoids like quercetin or luteolin glycosides) to the "poor antioxidant" compounds (monohydroxy B-ring-substituted flavonoids like apigenin glycosides; Agati et al., 2011). This was observed in olive leaves, where the increase of quercetin-3-*O*-glucoside and the decrease of apigenin-7-*O*-rutinoside resulted in an increased ratio of *o*-dihydroxy/monohydroxy B-ring-substituted flavonoids immediately after the UV-stress (~2x the ratio in control), suggesting that the flavonol

Table 2
Lipophilic composition (g kg^{-1} DW) of olive leaves from control (C), heat-shock (HS) and UV-B (UV-B) treatments immediately after stress experiment and 30 days after stress (HS and UV-B) relief. Values are given as mean ± standard deviation (n = 3–4). For each line and condition (stress exposure or stress relief), different letters indicate significant differences ($p < 0.05$). Retention time (Rt) is represented in minutes.

Rt	Compound	Stress Exposure			Stress Relief		
		C	HS	UV-B	C	HS	UV-B
Fatty Acid							
38.5	Palmitic Acid	0.669 ± 0.057^{a}	0.474 ± 0.054^{b}	0.669 ± 0.039^{a}	1.240 ± 0.040^{a}	0.598 ± 0.052^{b}	0.611 ± 0.015^{b}
42.3	Oleic Acid	0.660 ± 0.054^{a}	0.486 ± 0.050^{b}	0.689 ± 0.043^{a}	1.431 ± 0.055^{a}	0.708 ± 0.043^{b}	0.761 ± 0.037^{b}
Terpenes							
58.3	Thymol-β-D-glucopyranoside	0.037 ± 0.005^{a}	0.024 ± 0.008^{a}	0.050 ± 0.036^{a}	0.021 ± 0.003^{a}	0.027 ± 0.003^{a}	0.043 ± 0.007^{b}
66.9	β-Amyrin	0.134 ± 0.022^{ab}	0.094 ± 0.012^{b}	0.142 ± 0.017^{a}	0.189 ± 0.009^{a}	0.105 ± 0.007^{b}	0.110 ± 0.007^{b}
67.9	α-Amyrin	0.141 ± 0.024^{a}	0.102 ± 0.017^{b}	0.146 ± 0.014^{a}	0.200 ± 0.009^{a}	0.112 ± 0.009^{b}	0.114 ± 0.008^{b}
68.0	Lupeol derivatives 1	0.233 ± 0.062^{ab}	0.171 ± 0.038^{b}	0.270 ± 0.014^{a}	0.396 ± 0.025^{a}	0.343 ± 0.035^{b}	0.268 ± 0.020^{c}
70.1	Lupeol derivatives 2	0.243 ± 0.074^{a}	0.144 ± 0.038^{a}	0.266 ± 0.051^{a}	0.256 ± 0.025^{a}	0.186 ± 0.024^{b}	0.146 ± 0.010^{b}
71.1	Ursolic acid	0.087 ± 0.008^{a}	0.054 ± 0.007^{b}	0.082 ± 0.006^{a}	0.139 ± 0.008^{a}	0.053 ± 0.004^{c}	0.065 ± 0.001^{b}
Sterols							
60.5	Stigmast-5-en-3-ol	0.109 ± 0.011^{a}	0.073 ± 0.010^{b}	0.102 ± 0.010^{a}	0.149 ± 0.005^{a}	0.065 ± 0.004^{b}	0.072 ± 0.002^{b}
61.5	β-Sitosterol	0.186 ± 0.036^{ab}	0.140 ± 0.023^{b}	0.205 ± 0.023^{a}	0.271 ± 0.024^{a}	0.181 ± 0.013^{b}	0.172 ± 0.004^{b}

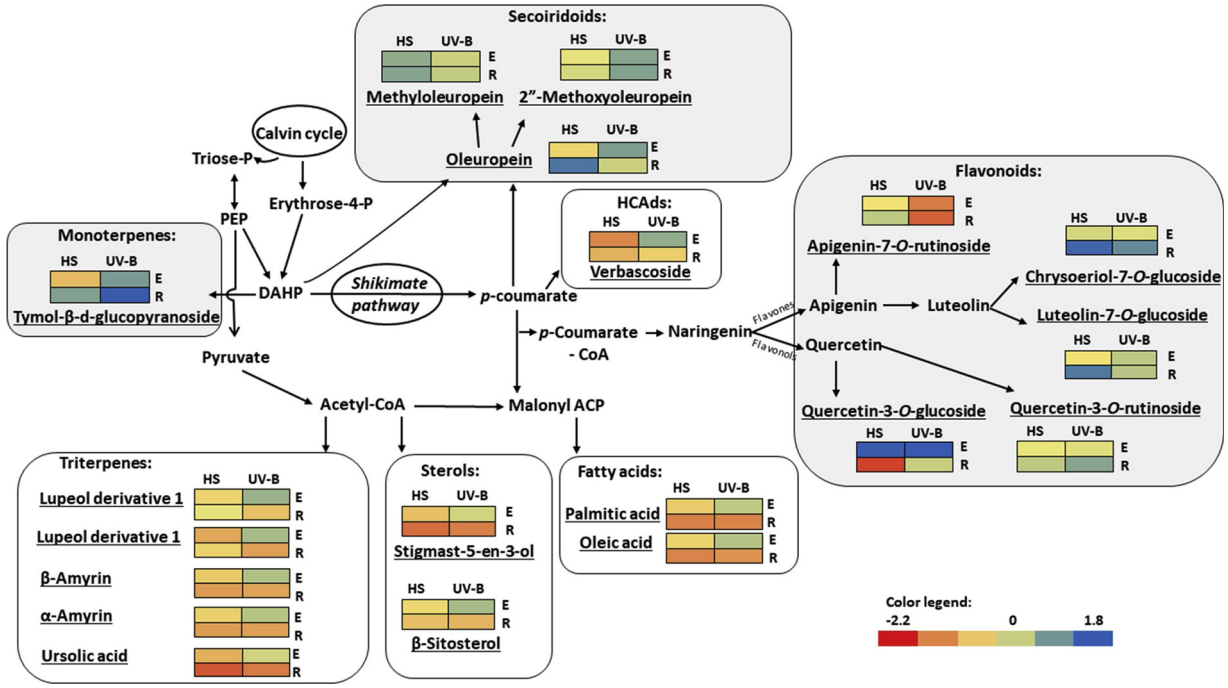

Fig. 1. General overview of the metabolites/compounds change in olive leaves immediately and 30 days after HS and UV-B treatments. The metabolites/compounds identified in Cobrançosa leaves are underlined. Relative levels [expressed as log2 (stress/control)] are given besides each identified metabolite/compound as a heat-map: HS – heat shock treatment; UV-B - high UV-B radiation treatment; E – exposure to stress treatments HS or UV-B; R – recovery from the HS and UV-B treatments (30 days after stress relief). Big grey squares contain groups of metabolites/compounds that respond more heterogeneously to the treatments (presenting increases, decreases or no changes).

quercetin-3-*O*-glucoside plays an important protection role against UV-B. Being flavones and flavonols originated by the same precursor naringenin, the change of this antioxidant ratio suggests that UV-B increases quercetin-3-*O*-glucoside synthesis in detriment of flavones synthesis (e.g. apigenin-7-*O*-rutinoside; Fig. 1). Moreover, this decrease of apigenin-7-*O*-rutinoside level may be due to an increase of its degradation or of its precursors. After stress relief, quercetin-3-*O*-glucoside role as an antioxidant seems to be replaced by other *o*-dihydroxy B-ring-substituted flavonoid like quercetin-3-*O*-rutinoside and the chrysoeriol-7-*O*-glucoside (Fig. 1, increases were not statistically significant), still remaining high the ratio of these compounds versus monohydroxy B-ring-substituted flavonoids. UV-B stimulated the synthesis of flavonols (e.g. quercetin) in privet shrub, grapevine, petunia and Arabidopsis plants, but in Deschampsia sp. and bushgrass, flavones (e.g. luteolin) and their biosynthetic precursors were preferably stimulated (Ferreyra et al., 2012). However, those studies did not follow the profile of those compounds after stress relief.

This is the first report about the changes in the most relevant flavonoids levels in olive plants during and after stress relief. Whilst our data pinpoint an increase on flavonols immediately after this stress, thus confirming that these metabolic adjustments are species dependent, it remains to understand why olive plants invest preferably in flavonols and flavones during the recovery to achieve homeostasis.

In the HS-stressed plants, the *o*-dihydroxy/monohydroxy B-ring-substituted flavonoids ratio followed a pattern different from that obtained for UV-B. Even though HS stimulates the levels of quercetin-3-*O*-glucoside, its contribution to this antioxidant ratio was neutralised by the decrease of luteolin-7-*O*-glucoside, while the monohydroxy B-ring-substituted flavonoid levels was maintained (Fig. 1). This reduction in luteolin-7-*O*-glucoside content may show its degradation, or its increased use in scavenging radicals (Agati et al., 2011), not being their product detected. Flavones also decreased in citrus plants under HS (Zandalinas et al., 2016), while in HS-stressed lettuce both flavonols (luteolin-7-*O*-glucoside) and flavones (quercetin-3-*O*-glucoside) levels increased (Oh et al., 2009). Unlike what was observed in olive, Arnica plants (a species adapted to low temperature) decreased the ratio of *o*-dihydroxy/monohydroxy B-ring-substituted flavonoids with the increase of temperature (Albert et al., 2009). Thus, the antioxidant ratio may be differently conditioned by the edaphoclimatic conditions to which the species is adapted. Interestingly, during HS recovery, the antioxidant ratio was increased (˜ 4x compared to control), mostly due to the strong accumulation of luteolin-7-*O*-glucoside. Our data show that flavonoids that are important during HS recovery differ from those mobilized during the stress. Unlike to UV-B treatment, HS seems to induce a later response with the increase of luteolin-7-*O*-glucoside and chrysoeriol-7-*O*-glucoside content. Whilst the role of these compounds during recovery deserves further studies, this increase may be related to its radical scavenger properties (Agati et al., 2011).

Secoiridoids, namely oleuropein and derivatives, also responded to UV-B and HS. The antioxidant importance of oleuropein as a strong radical scavenger relies on its two hydroxyl groups linked on the *o*-dihydroxy position to the A-ring (e.g. $O_2^{-\cdot}$, H_2O_2 and HO- species; Hassen et al., 2015). Also, the increase of oleuropein was related with plant oxidative damage and pathogen protection (Petridis et al., 2012; Daane and Johnson, 2010). Additionally, the increase of its derivative 2″-methoxyoleuropein (Michel et al., 2015) in response to UV-B, may be justified through oleuropein oxidation (the position of carbon C-2″ is reactive due to the intermediate stabilization). These data support that oleuropein plays an immediate leaf defence strategy against UV-B. Also, during recovery, the UV-B stressed plants show an evident capacity to reach homeostasis (Fig. 1), restoring the levels of these compounds close to those of the control. On the other hand, HS may promote oleuropein degradation (e.g. used as a reactive oxygen species scavenger; Kruk et al., 2005), or compromise its synthesis. Interestingly, the HS recovered plants showed an increase of oleuropein, suggesting that this compound's protective role may be more relevant during plant reestablishment.

Verbascoside is the most abundant hydroxycinnamic acid derivative in olive. This compound ranges from 0.3 to 29×10^3 mg kg^{-1} dry extract (Talhaoui et al., 2015), but its levels are influenced by seasonal

variations, plant life-cycle and nutritional status (Soler-Rivas et al., 2000). The two catechol groups (e.g. four hydroxyls in *o*-positions) confer the verbascoside a strong antioxidant capacity (D'Imperio et al., 2014). However, the negligible changes (Fig. 1) of this compound in stressed leaves suggest that verbascoside may not be relevant in olive protection against UV-B and HS, unlike to what was suggested by Pfündel et al. (2007).

The decrease of most triterpenes, sterols and fatty acids levels, was evident in HS, and aggravated during recovery of both HS and UV-B. Dias et al. (2018) also reported that the response profile of these compounds depended on the type of stress and its intensity. The functional relevance of fatty acids and sterols decreases deserves further attention. It may result from a direct damage of the stressor, or their catabolism be important as: a) a source for energy availability and/or for membrane integrity maintenance; and b) a precursor for the synthesis of other compounds like steroidal hormones and vitamins (Georgieva et al., 2011; Lukić et al., 2013). The profile of fatty acids and sterols of Cobrançosa under control conditions differ from those of Ascolano, Arbosa, Koroneiki and Grappolo cultivars (Cavalheiro et al., 2015), but is very close to the Galega (Dias et al., 2018).

Olive leaves are rich in terpenes and its content varies accordingly to the cultivar and growth conditions. In Cobrançosa, the most abundant were the monoterpene thymol-β-D-glucopiranoside, and the triperpenes β- and α-amirin, lupeol derivatives and ursolic acid. Other triterpenes, such as oleanolic acid, maslinic acid, uvaol, and erythrodiol, were identified in high amounts in other cultivars (Guinda et al., 2010; Peragón, 2013). In this work, only thymol-β-D-glucopiranoside increased during UV-B recovery, probably acting as an antioxidant (Dias et al., 2018).

5. Conclusions

We have characterized the levels of the most representative lipophilic and phenolic compounds of Cobrançosa leaves, and proved how these levels are modulated by UV-B and HS exposure and stress-recovery. Flavonoids, secoiridoids and monoterpenes levels are highly influenced by UV-B and HS, while triterpenes, sterols and fatty acids are less responsive. Both UV-B and HS treatments increase quercetin-3-*O*-glucoside, and UV-B stimulated oleuropein levels, suggesting that they may act as protective antioxidants against these conditions. HS decreased the levels of verbascoside, oleuropein and luteolin-7-*O*-glucoside, while in UV-B treatment only apigenin-7-*O*-rutinoside decreased. Thirty days after stress relief (recover) this profile of compounds changed. The levels of thymol-β-D-glucopyranosideas increased in UV-B recovered plants while oleuropein, luteolin-7-*O*-glucoside and chrysoeriol-7-*O*-glucoside levels increased in HS recovered plants. Other compounds, which were stimulated immediately after stress (e.g. quercetin-3-*O*-glucoside) in general decreased during plant recovery. This work demonstrates that olive leaves can be enriched in bioactive compounds in response to environmental conditions. Moreover, it remains to study if natural changes (due to climate change) or controlled manipulated of industrial orchards' growth conditions will similarly increase these leaf bioactive compounds to economically/industrial relevant levels, valorising this by-product.

Contributions

MC Dias planed and coordinated the experiments; C Figueiredo performed the metabolomic analysis and together with MC Dias, DCGA Pinto and AMS Silva analysed the results; MC Dias wrote the manuscript with contributions of all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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